# USE OF L-ISOASPARTYL METHYLTRANSFERASE AS LONGEVITY MARKER IN SEEDS

The present invention relates to the use of the L-isoaspartyl methyltransferase protein as a marker for the aging of seed batches.

Seed aging, which occurs during their storage, is accompanied by cell membrane deterioration, modification of the catalytic activity of numerous enzymes (for a revue, cf. Walters, C., 1998; *Seed Sci. Res.*, 8: 223-244), and the accumulation of DNA mutations. All these phenomena lead to a decrease in the germination vigor and the viability of the seeds.

The rapidity of the aging depends on a complex set of intrinsic factors (in particular genetic factors) and extrinsic factors (storage conditions). Since the degree of aging conditions the germination quality of the seeds, and also their subsequent storage capability, it is desirable to be able to determine it as rapidly and as precisely as possible.

The tests currently available for evaluating the germination quality of a seed batch are mainly tests based on germination trials taking into account various parameters, such as the mean germination time of the batch, the spreading out of the germination over time, the germination capability and the frequency of normal plantlets. The germination capability is, for example, defined by the percentage of normal plantlets that have germinated, after a given number of days after sowing, weighted by the percentage of very young shoots. These tests are defined and calibrated for each species or variety by means of a seed certification organism according to the ISTA (International Seed Testing Association) standards. However, these trials are

laborious to carry out. Furthermore, they make it possible to evaluate the current germination quality of a seed batch, but not to predict its future evolution, and in particular do not in any way make it possible to anticipate any drop in germination vigor.

At the current time, no specific markers are available for simply and accurately predicting the viability of a seed batch, and, *a fortiori*, its storage capability.

Among the many enzymes whose activity appears to be modified during aging (cf., Walters, 1998, *Seed Sci. Res.*, 8: 223-244 mentioned above), the inventors were more particularly interested in L-isoaspartyl methyltransferase (IAMT).

L-isoaspartyl methyltransferase (EC 2.1.1.77) is a protein repair enzyme involved in the conversion of abnormal L-isoaspartate and D-aspartate residues, present in proteins, to L-aspartate residues.

The L-isoaspartate and D-aspartate residues which constitute the substrate for L-isoaspartyl methyltransferase result from the spontaneous deamidation, isomerization and racemization of aspartate and asparagine residues, which occur during seed aging, and which are part of the modifications that can affect the functionality of the proteins into which these residues are integrated.

The L-isoaspartyl methyltransferase (IAMT or PIMT) makes it possible to convert at least some of these residues to L-aspartate residues, allowing the proteins thus repaired to recapture their activity.

L-isoaspartyl methyltransferase is a virtually universal enzyme which is found in a very large number

of organisms, from bacteria (Gram-negative) to plants and animals (O'Conner C.M. *et al*, 1985; *Biochem. Biophys. Res.*, 132: 1144-1150).

In plants, L-isoaspartyl methyltransferase activity appears to be present in virtually all plant species; L-isoaspartyl methyltransferase activity was in particular demonstrated in wheat germ (Trivedi L. *et al.*, *Eur. J. Biochem.*, 1982, 128: 349-354, Mudgett M.B. *et al, Biochemistry* 1993, 32: 11100-11111, *J. Biol. Chem.*, 1994, 269(41): 25605-25612), then in lotus (Shen-Miller *et al.*, *Am. J. Bot.*, 1995, 82(11): 1367-1380), *Arabidopsis thaliana* (Mudgett M.B. *et al, Plant. Mol. Biol.*, 1996, 30: 723-737; Thapar N. *et al, Protein Expr. Purif.*, 2000, 20: 237-251), barley (Mudgett M.B. *et al.*, *Plant Physiol.*, 1997, 115: 1481-1489), tomato (Kester *et al.*, *J. Exp. Bot.*, 1997, 309: 943-949), maize, carrot and rice (Thapar N. *et al.*, *Plant Physiol.*, 2001, 125(2): 1023-1035).

This activity is detectable in many organs in plants, but it appears to be at a maximum in dry seeds (Mudgett M.B. *et al.*, *Plant. Mol. Biol.*, 1996, 30: 723-737). Clarke S. (*Int. J. Pept. Protein Res.*, 1987, 30: 808-821) has shown *in vitro* that IAMT has a repair activity of approximately 30% of abnormal residues; the correct functioning thereof would therefore allow the seed or the plant to slow down the loss of integrity of proteins accumulating L-isoaspartate amino acids, but not to entirely compensate for the phenomena of racemization and isomerization. Shen-Miller *et al. (Am. J. Bot.*, 1995, 82(11): 1367-1380) have assayed the IAMT activity in sacred lotus (*Nelumbo nucifera*) seeds approximately 90 years old. The seeds that were found to be capable of germinating had high IAMT activity and the amount of racemized residues was equivalent to that of fresh seeds, i.e. 2%. Kester *et al.*, *(J. Exp, Bot.*, 1997, 309: 943-949) have reported, in artificially aged

tomato seeds, a decrease in IAMT activity correlated with a decrease in germination capability. Mudgett M.B. *et al.* *(Plant Physiol.,* 1997, 115: 1481-1489) have also observed, on naturally aged barley seeds, that the L-isoaspartyl methyltransferase activity decreases as a function of seed age and that this decrease is accompanied by the accumulation of racemized L-iso-aspartyl residues and also by a loss of seed viability; on the other hand, in the case of the artificially aged barley seeds, they have also observed an accumulation of racemized residues and a decrease in germination capability, but no decrease in IAMT activity.

These studies do not, however, make it possible to conclude that a direct correlation exists between the amount of L-isoaspartyl methyltransferase and the germination capability of seeds, which would make it possible to use this protein as marker for germination capability.

Furthermore, the results described in the studies mentioned above are based either on IAMT activity tests or on the detection of L-isoaspartyl methyltransferase mRNA.

Now, the assaying of the enzymatic activity makes use of laborious methods requiring the use of radioactive methanol, which are difficult to carry out in the context of routine control. Furthermore, as regards the mRNA, it has been observed that there is not always a correlation between the detection of IAMT transcripts and IAMT enzymatic activity. Thus, Mudgett M.B. *et al.* *(Plant. Mol. Biol.,* 1996, 30: 723-737) have reported that, in *Arabidopsis thaliana*, IAMT mRNA is detectable in the plantlets (where the enzymatic activity is not detectable), but not in the dry seed (where the enzymatic activity is at a maximum).

In *Arabidopsis thaliana*, two genes encoding L-isoaspartyl methyltransferases, the activity of which has been shown *in vitro*, have been identified at the current time. The first of them was described by Mudgett *et al.* (1996, mentioned above); the second was described by Xu Q. *et al.* (*Plant Physiol.*, 2004, 136: 1-13); these two genes were named, by the latter team, *PIMT-1* and *PIMT-2*, and their translation products were respectively named PIMT-1 and PIMT-2.

These two genes *PIMT-1* and *PIMT-2* are respectively located on chromosomes 3 and 5 of the *Arabidopsis* genome. However, at the current time, the involvement of these two genes in the enzymatic activities measured *in vivo* has not been described.

The inventors have succeeded in identifying a *PIMT-1* gene regulation mutant in *Arabidopsis thaliana* and have thus been able to note that the expression of this gene plays an essential role in the storage capability of the dry seed during storage. They have also identified, from the PIMT-1 protein, a peptide that makes it possible to obtain antibodies capable of differentiating PIMT-1 and PIMT-2 in *Arabidopsis thaliana*, and of recognizing PIMT-1 orthologs in other dicotyledons, and a peptide that makes it possible to obtain antibodies that recognize PIMT-1 orthologs in other dicotyledonous or monocotyledonous plant species.

The quantification of L-isoaspartyl methyltransferase using antibodies obtained against one or other of these peptides has made it possible to establish a correlation between the disappearance of the protein in the dry seed and the loss of germination vigor. Furthermore, the inventors have observed that the decrease in the amount of L-isoaspartyl methyltransferase precedes the decrease in germination vigor. It therefore appears that this enzyme constitutes a predictive marker which

makes it possible not only to evaluate the germination capability of seeds, but also to predict a decrease in this capability during storage.

A subject of the present invention is a method of determining the germination vigor and/or the storage capability of a seed batch, characterized in that it comprises quantifying, on a sample of seeds taken from said batch, the proteins recognized by anti-L-isoaspartyl methyltransferase antibodies directed against a region of said protein defined by the sequence (I): RYVPLTSR$X_1X_2$QL$X_3$ (SEQ ID NO: 1), in which $X_1$ represents E, V or S, $X_2$ represents A or E, and $X_3$ represents R, G or Q.

The antibodies directed against a peptide of sequence (I) recognize the L-isoaspartyl methyltransferases PIMT-1 and PIMT-2 in *Arabidopsis thaliana*, and also orthologs of these proteins in other dicotyledonous or monocotyledonous plant species.

The method in accordance with the invention can advantageously be carried out by immunoassay. In this case, use may be made of anti-L-isoaspartyl methyltransferase antibodies directed against a peptide of sequence (I); use may also be made of anti-L-isoaspartyl methyltransferase antibodies directed against another region of the protein, in particular the region defined by the sequence (II): Q$X_4$L$X_5$V$X_6$DK$X_7X_8$DGS$X_9X_{10}X_{11}$ (SEQ ID NO: 2), in which $X_4$ represents D or E, $X_5$ represents Q or K, $X_6$ represents V or I, $X_7$ represents N or S, $X_8$ represents S, E or A, $X_9$ represents either a dipeptide chosen from IS, VS, VT and TS, or a peptide bond, $X_{10}$ represents I or V, and $X_{11}$ represents K, Q or R.

Q, D, G, S, V, R, Y, V, P, L, T, S, R, K, I, E, A, N, H and F here have their usual 1-letter code meaning.

For example, in order to differentiate PIMT-1 and PIMT-2 in *Arabidopsis thaliana*, use may be made of antibodies directed against the region defined, in the *Arabidopsis thaliana* PIMT-1 protein, by the sequence QDLQVVDKNSDGSVSIK (SEQ ID NO: 3), or antibodies directed against the corresponding region of the *Arabidopsis thaliana* PIMT-2 protein, which is defined by the sequence QELKVIDKNEDGSIK (SEQ ID NO: 4).

Antibodies directed against the region defined by sequence (II) of a dicotyledon L-isoaspartyl methyltransferase can also be advantageously used for carrying out a method in accordance with the invention on dicotyledon seeds. For example, antibodies directed against the region of the *Arabidopsis thaliana* PIMT 1 protein defined by the sequence QDLQVVDKNSDGSVSIK (SEQ ID NO: 3), or against the homologous region of PIMT-1 orthologs in other dicotyledons, can also be advantageously used for carrying out a method in accordance with the invention on dicotyledon seeds.

Similarly, antibodies directed against the region defined by sequence (II) of a monocotyledon L-isoaspartyl methyltransferase can be used for carrying out a method in accordance with the invention on monocotyledon seeds.

A subject of the present invention is also any anti-isoaspartyl methyltransferase antibody directed against a region of said protein chosen from the region defined by sequence (I) and the region defined by sequence (II) above.

By way of nonlimiting examples of anti-isoaspartyl methyltransferase antibodies directed against a region defined by sequence (I), mention may be made of: antibodies directed against the region of sequence

RYVPLTSREAQLR (SEQ ID NO: 5) of *Arabidopsis thaliana* PIMT-1; antibodies directed against the region of sequence RYVPLTSRVEQLG (SEQ ID NO: 6) of *Arabidopsis thaliana* PIMT-2; antibodies directed against the region of sequence RYVPLTSRSAQLQ (SEQ ID NO: 7) of a wheat PIMT; antibodies directed against the region of sequence RYVPLTSRSAQLQ (SEQ ID NO: 7) of a rice PIMT.

By way of nonlimiting examples of anti-isoaspartyl methyltransferase antibodies directed against a region defined by sequence (II), mention may be made of: antibodies directed against the region of sequence QDLQVVDKNSDGSVSIK (SEQ ID NO: 3) of *Arabidopsis thaliana* PIMT-1; antibodies directed against the region of sequence QELKVIDKNEDGSIK (SEQ ID NO: 4) of *Arabidopsis thaliana* PIMT-2; antibodies directed against the region of sequence QDLQVIDKSADGSTSVR (SEQ ID NO: 8) of a wheat PIMT; antibodies directed against the region of sequence QELQVVDKNADGSVTVQ (SEQ ID NO: 9) of a rice PIMT.

The expression "antibody directed against a region of an L-isoaspartyl methyltransferase" here defines any antibody capable of binding in a detectable manner with the region concerned, on the whole protein or on a fragment thereof, but which does not bind in a detectable manner with another region of the same protein. In the case of monoclonal antibodies, this includes any antibody that recognizes an epitope located in said region; in the case of polyclonal antibodies, this includes any antibody preparation that does not exhibit any significant cross reactions with other regions of the L-isoaspartyl methyltransferase.

On the other hand, this antibody can exhibit a more or less sizable percentage of cross reactions with the homologous region of the L-isoaspartyl methyltransferases of other plant species. In particular, as

indicated above, antibodies directed against the region defined by sequence (I) generally exhibit a very broad specificity and can recognize a large variety of L-isoaspartyl methyltransferases in monocotyledons or dicotyledons, whereas the antibodies directed against the region defined by sequence (II) generally exhibit a more restricted specificity.

Antibodies in accordance with the invention can be obtained by methods well known in themselves. Conventionally, a fragment of L-isoaspartyl methyltransferase (in the form of natural, recombinant or synthetic peptide) comprising the region against which it is desired to direct the antibody, or at least one fragment thereof that is sufficient in size to constitute a B epitope (generally at least 5 to 7 amino acids) is used as immunogen. If necessary, said peptide is mixed with an adjuvant, or coupled to a carrier protein, in order to increase its immunogenicity. The antibodies obtained can then be purified, in a manner also known in itself. Generally, this purification comprises at least one step consisting of affinity chromatography on a column onto which is grafted the peptide against which the antibody must be directed.

By way of nonlimiting examples, antibodies directed against the region of an L-isoaspartyl methyltransferase defined by sequence (I) can be obtained using, as immunogen and/or for the purification of the antibodies by affinity chromatography, the peptide of sequence RYVPLTSREAQLR (SEQ ID NO: 5), which corresponds to this region in *Arabidopsis thaliana* L-isoaspartyl methyltransferase PIMT-1; use may also be made, in the same manner, of peptides comprising a fragment of this sequence, for example the fragment of sequence RYVPLTSR (SEQ ID NO: 10), or the fragment of sequence RYVPLTSREAQL (SEQ ID NO: 11); use may also be made of longer fragments of PIMT-1, for example a peptide of

sequence RYVPLTSREAQLRGD (SEQ ID NO: 12).

A subject of the present invention is also a method of quantifying the L-isoaspartyl methyltransferase in plant material, characterized in that it comprises bringing said material into contact with an anti-L-isoaspartyl methyltransferase antibody in accordance with the invention.

A subject of the present invention is also the use of an anti-L-isoaspartyl methyltransferase antibody in accordance with the invention, for determining the germination vigor and/or the storage capability of a seed batch.

The present invention will be understood more fully from the further description which follows, which refers to examples described in the demonstration of the correlation between the amount of L-isoaspartyl methyltransferase and the storage capability of seeds, and also the production of an anti-L-isoaspartyl methyltransferase antibody and the use thereof for evaluating the germination quality of seeds of various species.

**EXAMPLE 1: IDENTIFICATION AND CHARACTERIZATION OF AN L-ISOASPARTYL METHYLTRANSFERASE REGULATION MUTANT IN *ARABIDOPSIS THALIANA***

Screening of the *Arabidopsis thaliana* T-DNA transformant collection of the INRA [French National Institute for Agricultural Research] in Versailles made it possible to isolate an insertion mutant in the *PIMT-1* gene. This mutant was named *atpcm1.1*⁻. The sequencing data show that the T-DNA insertion is located 173 base pairs upstream of the ATG, in the promoter region of the L-isoaspartyl methyltransferase gene, where it caused a deletion of 13 base pairs,

bringing about the disappearance of an abscissic acid-response element box (ABRE box).

Mutant plants homozygous for the T-DNA insertion were selected for studying the properties of the mutant.

**Expression of the *PIMT-1* gene in the *atpcm1.1*$^-$ mutant**

The expression of the *PIMT-1* gene in the plantlets and in the dry seeds in the *atpcm1.1*$^-$ mutant, and in the wild-type plant, was compared by semi-quantitative RT-PCR. By way of a control, the EF transcription factor gene, which exhibits a constitutive expression profile during development in the *Arabidopsis thaliana* seed, is used.

The plantlets are obtained by sowing seeds on a nylon filter (Buisine, Nycom, 5 µm diameter mesh) lying on a 0.5% water-agar medium. After stratification (4°C, 2 days), the dishes are placed in a culture chamber for 4 days. The filters on which the plantlets are arranged are then transferred onto a water-agar medium for one day, before the RNAs are extracted.

The total RNAs are extracted from the plantlets or from the dry seeds according to the hot phenol technique of Verwoerd *et al.* (*Nucleic Acids Res.*, 1989, 17(6): 2362), modified by the addition of a second precipitation with LiCl (2M final concentration) for 6 hours. The purification of the poly(A)$^+$ is carried out from the total RNAs using oligo(dT)$_{25}$ attached to Dynabead® magnetic beads (Dynal).

The first cDNA strand is synthesized by reverse transcription (RT) of the poly(A)$^+$ mRNAs.

The PCR amplification is carried out on 10 ng of the RT product.

For the L-isoaspartyl methyltransferase gene, one of the following pairs of primers (specific for the *PIMT-1* gene) is used:

AMEAs:
GCT ATG GAG GCT GTG GAT AGA GG (SEQ ID NO: 13);
AQLRa:
TCA GTC CCC TCT CAG CTG CGC (SEQ ID NO: 14);
or
GTGYs:
GGA CCG GGT ACT TAA CTG CTT (SEQ ID NO: 15);
and AQLRa,
and for the EFα elongation factor gene, the following pair of primers is used:
F1αA4-low:
TTG GCG GCA CCC TTA GCT GGA TCA (SEQ ID NO: 16);
F1αa4-up:
ATG CCC CAG GAC ATC GTG ATT TCA T (SEQ ID NO: 17).

The PCR is stopped at 15, 18 or 21 cycles in order to anticipate the moment at which the amplification is no longer linear, and therefore no longer interpretable in terms of initial amounts.

The PCR products are loaded onto an agarose gel (0.8%) and then transferred, in an alkaline medium (0.4N sodium hydroxide) onto a nylon membrane, and the latter is hybridized with a radiolabeled probe specific for the *PIMT* gene (IAMT probe) or with a radiolabeled probe specific for the *EF* gene.

The hybridization signal is quantified with the MacBas® software.

**Results obtained in the 5-day plantlets**:

These results are illustrated in figure 1.

Legend of figure 1:
(A) electrophoresis gel;
(B) autoradiograph after hybridization with the probe;
(C) signal quantification;
WT: wild-type plantlets; *Atpcm*: plantlets of the *atpcm1.1*$^-$ mutant;
a: 15 PCR cycles; b: 18 PCR cycles; c: 21 PCR cycles.

These results show that the gene is expressed 1.5 times more in the wild-type than in the mutant at the 5-day plantlet stage on water-agar.

This experiment was also carried out using plantlets which, after having spent 4 days in a culture chamber, were transferred onto a water-agar medium containing 100 µM of abscissic acid, one day before extraction of the RNAs.

The results are illustrated in figure 2:

Legend of figure 2:
(A) autoradiograph after hybridization with the probe;
(B) signal quantification;
WT: wild-type plantlets; *Atpcm*: plantlets of the *atpcm1.1*$^-$ mutant;
a: 15 PCR cycles; b: 18 PCR cycles; c: 21 PCR cycles.

These results show that the wild-type plantlets overexpress the *IAMT* gene in the presence of ABA, which confirms the observations previously made by Mudgett M.B. *et al.* (*Plant Mol. Biol.*, 1996, 30, 723-737, mentioned above), and that they accumulate 3 times more IAMT transcripts than the *atpcm1.1*$^-$ plantlets. The *atpcm1.1*$^-$ mutant visibly lost its ability to induce the expression of the IAMT in the presence of ABA in the plantlets. This data makes it possible to confirm that the region deleted by the mutation clearly contains a regulatory sequence (ABRE

box) involved in the ABA response.

**Results obtained in the dry seeds**:

The results are illustrated in figure 3.

Legend of figure 3:
(A) autoradiography after hybridization with the probe;
(B) signal quantification;
WT: wild-type seeds; *Atpcm*: seeds of the *atpcm1.1*$^-$ mutant;
a: 15 PCR cycles; b: 18 PCR cycles; c: 21 PCR cycles.

No accumulation of the IAMT transcript is observed in the dry seeds of the wild-type plant, which confirms the observations previously made by Mudgett M.B. *et al.* (*Plant Mol. Biol.*, 1996, 30, 723-737, mentioned above). On the other hand, the *atpcm1.1*$^-$ mutant accumulates the IAMT transcript at a not insignificant level in the dry seed.

**EXAMPLE 2: INFLUENCE OF ACCELERATED AGING ON THE GERMINATION VIGOR OF THE SEEDS OF WILD-TYPE *ARABIDOPSIS THALIANA*, OR OF THE *ATPCM1.1*$^-$ MUTANT**

Accelerated aging experiments were carried out on freshly harvested seeds in order to test whether a difference in viability exists between the wild-type and the *atpcm1.1*$^-$ mutant. The aging conditions chosen very gradually affect the germination vigor of the seeds so as to then result in a loss of viability. The choice of mild conditions mimics the natural aging conditions and makes it possible to finely analyze the impact of the IAMT in the evolution of the germination vigor during aging.

The production of seeds of the mutant and of the wild-type control is carried out by cultivating the mother

plants at the same time and in the same chamber. The accelerated aging is carried out at 40°C (Jouan incubator) and 48% RH (obtained with saturated $Mg(NO_3)_2$ salt) (hermetic jar and seeds in sterile filter paper sachets). The temperature and the humidity are controlled with a Testostor 175 (Testo).

Samples are taken after 2, 6 and 9 weeks of aging and a germination test is carried out on 200 seeds.

The germination takes place in a Petri dish (55 mm diameter) on an aqueous medium supplemented with MES (2-N-morpholinoethanesulfonic acid) at 0.58 g/l, adjusted to pH 5.9, and solidified with agar (7 g/l). The sowing is carried out under a laminar flow hood, at a rate of 25 seeds per dish, arranged evenly on the medium so as to be able to detect the beginning of germination more readily. The seeds sown are observed daily with a magnifying lens. A seed is considered to have germinated when the emergence of the radicle is visible with a magnifying lens.

Each trial comprises 8 repetitions of 25 seeds and the means of the percentages of germinated seeds after 3 days of soaking are reported on the graph. A seed is said to be germinated when the radicle is visible.

The germination is carried out in a Petri dish (55 mm diameter), at a rate of 25 seeds per dish, on a solid aqueous medium (7 g/l agar) supplemented with 0.58 g/l of MES (2-N-morpholinoethanesulfonic acid) and adjusted to pH 5.9.

This test is carried out under various temperature conditions: (1) an alternation of temperatures 20-25°C, which corresponds to the optimal conditions for germination and makes it possible to measure the seed viability; (2) a temperature of 15°C, which is slightly

disadvantageous for germination; a decrease in the germination capability under these conditions reflects a decrease in germination vigor of the seed batch. The results are illustrated in figure 4.

Legend of figure 4: germination capability 3 days after sowing, at 20-25°C (A), and 15°C (B), as a function of the duration of storage.

- ♦ wild-type seeds;
- ■ seeds of the *atpcm1.1*⁻ mutant.

The kinetics of germination carried out with a 20-25°C temperature alternation (figure 4 A) do not show any significant differences in the germination capability of the seeds of the mutant and of the wild-type. On the other hand, when the seeds are soaked at 15°C (figures 4 B), which is a temperature at which differences in germination vigor can be demonstrated, it appears that the germination vigor of the mutant-type seeds, based on 6 weeks of accelerated aging, is approximately 30% greater than that of the wild-type seeds. This experiment was reproduced 3 times with independent biological samples and gave the same result.

**EXAMPLE 3: PRODUCTION OF ANTI-IAMT ANTIBODIES**

2 peptides of the C-terminal region of the *Arabidopsis thaliana* isoaspartyl methyltransferase-1: QDLQVVDKNSDGSVSIK (peptide 1, SEQ ID NO: 3), and: RYVPLTSREAQLR (peptide 2, SEQ ID NO: 5) were chosen as immunogens.

The antibodies were prepared in rabbits according to the following protocol:

After a 1.5 ml sample of "preimmune" serum has been taken, the rabbits are immunized with a first injection

of the chosen peptide (4 mg in solution as 200 µg of peptide in 5 ml of a solution containing the carrier protein KLH mixed with 5 ml of Freund's adjuvant), followed by booster injections 1, 2, 4, 8 and 15 weeks after the 1st injection. The immune serum (50 ml) is taken 20 weeks after the 1st injection.

The antibodies are purified by affinity chromatography on a column of cyanogen bromide-activated Sepharose 4F (Pharmacia), grafted with 20 mg, per gram of Sepharose gel, of the peptide used as immunogen.

5 ml of serum diluted beforehand in 5 ml of 1× PBS (40 mM NaCl; 2.5 mM KCl; 8.1 mM $Na_2HPO_4$, 15 mM $KH_2PO_4$; pH 7.2) are applied to the column.

The gel is washed 3 times with 10 ml of 1× PBS. The elution is carried out by passing 10 ml of a solution of 0.2 M glycine/0.15 M NaCl, pH 2, over the column. The fractions eluted are collected in tubes containing 0.1 ml of Tris, pH 8.8 (1.5 M).

The fractions containing the antibody are combined (dialyzed against a solution of cold 1× PBS), and then supplemented with 0.02% of sodium azide. The fractions are stored at 4°C.

To verify the specificity of the antibody against the PIMT-1 protein, the cDNAs of the *PIMT-1* and *PIMT-2* genes were cloned separately into the expression vector pDEST17 (Invitrogen) under the control of the T7 bacterial promoter and downstream of a 6×His tag, and expressed in the *E. coli* strain BL21.

The recombinant PIMT-1 and PIMT-2 proteins expressed by *E. coli* were individually purified by affinity chromatography on nickel beads, and were analyzed by Western blotting with the antibody 1.

The results are given in figure 5.

Legend of figure 5:

Figure 5A represents Coomassie blue staining of an electrophoresis gel obtained from protein extracts of *E. coli* containing the PIMT-1 or PIMT-2 cDNA, or no cDNA (T-), in the expression vector pDEST17. The arrows indicate the presence of the *Arabidopsis thaliana* PIMT protein.

Figure 5B represents a Western blot carried out with the antibody 1 on the PIMT-1 and PIMT-2 proteins purified from the extracts presented in figure 6A.

These results show that the antibody 1 makes it possible to specifically reveal the *Arabidopsis thaliana* PIMT-1 protein.

**EXAMPLE 4: WESTERN BLOTTING DETERMINATION OF THE IAMT PROTEIN CONTENT IN THE DRY SEEDS OF *A. THALIANA*, AND OF THE EVOLUTION THEREOF DURING AGING**

The anti-IAMT antibodies generated, as described in example 3 above, against the peptide 1 (antibody 1) were used to determine the L-isoaspartyl methyltransferase protein content of wild-type or mutant-type *Arabidopsis thaliana* dry seeds that were freshly harvested or aged for 2, 6 and 9 weeks as described in example 2 above.

**Extraction of the seed proteins:**

The seeds are ground in liquid nitrogen until a fine powder is obtained. The powder is transferred to a third of a 2 ml Eppendorf tube completed with 2X Laemmli buffer (Laemmli U.K., *Nature*, 1970, 227: 680)

preheated to 100°C. The samples are incubated at 100°C for 5 minutes before being centrifuged at 4°C (6000 g, 5 minutes). The supernatant is recovered, aliquoted and stored at -20°C. Prior to use, 100 µl of supernatant are precipitated from an equivalent volume of 20% trichloroacetic acid (13 000 rpm, 5 minutes). The pellet is washed with 200 µl of 80% acetone, dried, and taken up in 1 ml of 0.2N sodium hydroxide. The concentration is determined by measuring the absorbance at 595 nm of the protein-Bradford reagent (Bio-Rad) complex. Bovine serum albumin is used to obtain a standard curve.

**Western blotting:**

The electrophoresis of the proteins (10 µg) is carried out under denaturing conditions (0.1% SDS) on a polyacrylamide gel (12%) in accordance with Laemmli (*Nature*, 1970, 227: 680). The semi-dry blotting is carried out on a nylon membrane of Hybond C type (Amersham) under a current of 20 V for 50 minutes. The membrane is then incubated for 40 minutes in a solution of PBS-T (1.4M NaCl; 27 mM KCl; 81 mM $Na_2HPO_4$, 150 mM $KH_2PO_4$; 0.001% Tween® 20) to which powdered milk (5%) is added in order to saturate the nonspecific sites.

The anti-IAMT primary antibody is added (1/5000th dilution) and incubation is carried out overnight at 4°C with shaking. After 3 washes for 10 minutes with PBS-Tween, the membrane is incubated with PBS-Tween® + milk to which the peroxidase-coupled anti-rabbit IgG secondary antibody (Bio-Rad) has been added, for 2 hours at ambient temperature with shaking. The membrane is again washed twice with PBS-Tween. The visualization is carried out by chemiluminescence, using the Covalight kit, under the conditions recommended by the manufacturer (CovalAb). After 1 minute, the membrane is placed under an X-ray film and exposed for 30 minutes.

The results are illustrated in figure 6.

Legend of figure 6: seeds of wild-type *Arabidopsis thaliana* (WT); seeds of the *atpcm1.1*$^-$ mutant (MT); suffix 2, 6 or 9: seeds aged for 2, 6 or 9 weeks.

In all the cases, a single band is observed. The protein recognized corresponds to the size expected for the PIMT-1 enzyme, which shows the specificity of the antibody 1 for this enzyme in the dried seed.

In the wild-type seeds, a decrease in the amount of L-isoaspartyl methyltransferase protein is observed, which manifests itself from 2 weeks of accelerated aging, and which continues up to the end of the experiment. Conversely, in the seeds of the *atpcm1.1*$^-$ mutant, an increase in the amount of enzyme is observed during the aging.

This observation corroborates the results reported in example 1, showing the accumulation of the transcript specific for the PIMT-1 gene in the dry seeds of the *atpcm1.1*$^-$ mutant, and can be correlated with the better germination vigor of the *atpcm1.1*$^-$ mutant after accelerated aging reported in example 2.

These data provide genetic proof that the accumulation of the PIMT-1 protein is associated with better seed longevity, and indicate that the L-isoaspartyl methyl-transferase protein constitutes an advantageous marker for the evolution of the germination vigor of a seed batch during storage.

**EXAMPLE 5: USE OF THE ANTI-IAMT ANTIBODY FOR DETERMINING THE STORAGE CAPABILITY OF MAIZE, RAPESEED, TOMATO AND BEAN SEEDS**

Complementary experiments were carried out in order to verify whether the antibody obtained against the *Arabidopsis thaliana* L-isoaspartyl methyltransferase also recognized the orthologous enzymes of other plant species, and whether IAMT also constituted, in these species, a marker for germination capability.

Several species of agronomic interest were chosen for these experiments in order to explore a broad spectrum of crop plants: maize is a representative of monocotyledons and, in dicotyledons, the bean is a leguminous plant, rapeseed is a cruciferous plant and the tomato is a solanaceous plant.

The accelerated aging is carried out by incubation at a given temperature after having equilibrated the seeds at a desired water content (generally 16 to 17%).

The seeds are first overdried at 20% RH (~ 28°C) in a ventilated incubator. To determine the water content, 10 g of seeds are coarsely ground and weighed before being heated to dryness (130°C, 1 hour). The seed powder is cooled under vacuum and re-weighed (the accuracy is to ten one thousandths of a gram). It is then possible to estimate the initial water content of the seeds after overdrying according to the formula:

$$\frac{M_i - M_f}{M_i} = WC_i$$

$M_i$ = mass of seeds before heating
$M_f$ = mass of seeds after heating
$WC_i$ = initial water content

In order to determine the mass of water to be added to a known mass of seeds in order to fix its water content at the desired value, the following formula is used:

$$\frac{M_1 (WC_i - WC_f)}{WC_f - 1} = M_2$$

$WC_i$ = initial water content
$WC_f$ = final water content
$M_1$ = mass of seeds
$M_2$ = final water mass

The seeds are placed in a leakproof container containing the calculated volume of water. The soaking is carried out for 3 days with shaking.

**Aging in the bean:**

2 bean seed batches (harvest 2000 = batch 178 and 2001 = batch 197) are equilibrated at 16% water content and placed in hermetic jars at 40°C. A sample is taken after 3, 7, 11 and 14 days of aging and a germination test is carried out on 50 seeds.

The results are illustrated in figure 7A.

Legend of figure 7A: germination capability, expressed per 100 seeds, as a function of the duration of the controlled degradation.

◊ batch 197
■ batch 178.

These results show that the 2 batches do not behave in the same way under similar degradation conditions, since batch 178 (harvest of 2000) reaches 45% germination after 3 days of aging, whereas this percentage of germination is reached in 9 days for batch 197 (harvest of 2001).

The proteins derived from the dry bean seeds, originating from the harvests of 2000 and 2001, and also from the artificially aged seeds from these 2 harvests, were also characterized by Western blotting using the antibody 1.

The results are illustrated in figure 7B:

Legend of figure 7B: 178 (harvest of 2000); 197 (harvest of 2001); (T3), (T7), (T11) days: 3, 7 and 11 days of aging.

The antibody 1 demonstrates 2 or 3 proteins having a size of approximately 46 kDa. It is observed that batch 197, which exhibits a better storage capability with accelerated aging than batch 178, contains a greater amount of proteins recognized by the antibody 1. With accelerated aging, batch 197 (harvest 2001) shows a decrease in the concentration of this set of proteins for the periods of aging which precedes the loss in viability. Batch 178 (harvest 2000), whose germination capability dropped from the 3rd day of aging, does not exhibit any substantial decrease in the amount of proteins recognized by the antibody 1.

**Aging in rapeseed:**

The seed batch (rapeseed Ontario, harvest 2002) is subjected to an accelerated aging treatment under the conditions described above on the bean. The samples are taken in the same manner at the same periods of aging and the germination capabilities (GC) of the various samples are illustrated in figure 8A.

Legend of figure 8A:
Germination capability, expressed per 100 seeds, as a function of the aging period. The dry mature seeds of a harvest from 2002 have a germination capability of 92% in 2003, and the germination capability of these seeds decreases to 91%, 74%, 3% and 0% over the times T0, T1, T2 and T3 of the aging treatment, respectively.

The proteins derived from the dry rapeseed seeds,

originating from the harvest of 2002, and also from the artificially aged seeds, were also characterized by Western blotting using the antibody 1.

The results are illustrated in figure 8B:

Legend of figure 8B:
The antibody 1 demonstrates 1 protein having a size of approximately 32 kDa. It is observed that the seed batch not having been subjected to aging treatment, which exhibits a better germination capability, contains a larger amount of proteins recognized by the antibody 1. Over the accelerated aging times T0, T1, T2 and T3, this batch shows a decrease in the concentration of this protein, which is proportional to the decrease in germination capability represented in figure 8A.

**Aging in the tomato:**

Two batches of tomato seeds (variety PicNic) from different harvest years (1994 and 2000) were analyzed in 2003 for their germination capability (illustrated in figure 9A). These 2 seed batches were therefore subjected to an aging of 9 years and of 3 years, respectively, under optimal storage conditions such as those conventionally used by seed producers.

Legend of figure 9A:
Germination capability (GC) in 2003, expressed per 100 seeds, as a function of the year of harvest (1994 and 2000). The seed batches harvested in 1994 and in 2000 have a germination capability of 89% and 96%, respectively.

The proteins derived from the dry tomato seeds, originating from these 2 years of harvest of 2002, were also characterized in 2003 by Western blotting using

the antibody 1.

The results are illustrated in figure 9B.

Legend of figure 9B:
The antibody 1 demonstrates 3 or 4 proteins having a size of approximately 24 kDa, 40 kDa and 52 kDa. It is observed that the batch harvested in 2000, which exhibits a better GC, contains a larger amount of proteins recognized by the antibody 1.

**Aging in maize:**

The germination capabilities of seed batches derived from various years of harvest (1996, 1999, 2000, 2001) were analyzed in 2002.

The results of this analysis are illustrated in figure 10A.

Legend of figure 10A:
Germination capability (GC) in 2002, expressed per 100 seeds, as a function of the year of harvest (1996, 1999, 2000 and 2001). Under the conventional storage conditions, the maize seed batches harvested in 2001 and 2000 give 100% germination, the seed batch harvested in 1999 gives 96% germination and the seed batch harvested in 1996 gives 75% germination.

In order to carry out the controlled degradation of the maize, the water content of the seed is fixed at 17%. The aging is carried out at 45°C. A sample is taken after 1, 3 and 5 days of aging and a germination test is carried out on 2000 seeds.

Under these conditions, a complete inability to germinate is obtained in 5 days, as shown in figure 10B.

Legend of figure 10B: Percentage germination as a function of the duration of controlled degradation.

The protein of the dry seeds derived from the harvests of 1996, 1999, 2000 and 2001, and also those of seeds harvested in 2001 and artificially aged, were extracted in 2003 and analyzed by Western blotting using the antibody 1 and the antibody directed against the peptide 2 (antibody 2) as described in example 4 above. The results are given in figure 10C (for the antibody 1) and 10D (for the antibody 2).

Legend of figures 10C and 10D: 96, 99, 00, 01: year of harvest; (T1), (T3), (T5) days: 1, 3 and 5 days of aging.

In figure 10C, no signal is detected with the antibody 1 on the protein extracts of maize, compared with the signals obtained on the protein extract of bean used as controls. This result indicates that the antibody 1 does not make it possible to detect the PIMT protein in maize. The same result (not shown here) was obtained in other monocotyledons such as wheat and onion. The antibody 1 shows specificity with respect to dicotyledons.

The use of the antibody 2 (figure 10D) makes it possible, on the other hand, to detect 2 proteins of molecular weights 40 kDa and 55 kDa in maize. These proteins cannot be detected in the seeds harvested in 1996, for which the percentage germination, measured in parallel, is 75%. The content of 40 kDa protein is at a maximum in the seeds derived from the harvest of 2001, and then it subsequently decreases with the age of the harvest. In the seeds from 1999, it is barely detectable, whereas the percentage germination of these seeds is still 96%. The content of 55 kDa protein remains, on the other hand, relatively stable between

the harvests of 1999 and of 2001.

With accelerated aging, the amount of 55 kDa protein remains stable during the first day of accelerated aging, and it is then no longer detectable. The 40 kDa protein is no longer detectable from the first day of accelerated aging. The same observations (results not shown) were made in wheat and onion).

It therefore appears that the disappearance of the 40 and 55 kDa proteins recognized by the anti-IAMT antibodies precedes the decline in germination capability. These proteins, and in particular the 40 kDa protein, can therefore be used as a predictive marker for aging of the seed. This analysis also shows that the antibody 2 makes it possible to follow the evolution of this protein also in monocotyledons, whereas the antibody 1 itself appears to be more specific for dicotyledons.

These results shows that anti-IAMT antibodies in accordance with the invention make it possible to demonstrate differences in storage capability between seed batches, both under natural storage conditions, such as those used by seed producers, and in a situation of controlled deterioration.